Lactobacillus rhamnosus GG: An Updated Strategy to Use Microbial Products to Promote Health

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Abstract

It is now widely appreciated that probiotics exert their beneficial effects through several mechanisms, including inhibitory effects on pathogens, maintenance of the balance of intestinal microbiota, and regulation of immune responses and intestinal epithelial homeostasis. A significant area of progress has come from observations that specific products derived from probiotics mediate their mechanism(s) of action. This review focuses on new insights into the well-studied probiotic bacterium Lactobacillus rhamnosus GG (LGG). The biologic consequences of LGG-derived products enhance LGG adherence to intestinal epithelial cells and protect intestinal epithelial cells from injury through regulating several signaling pathways. Thus, LGG-derived products may provide novel approaches for health and disease prevention and treatment, especially for intestinal inflammatory disorders. However, compared to LGG functional proteins predicted by analysis of LGG genome sequences, the number of identified LGG-derived products is limited. As more mechanistic evidence becomes available to characterize the relationship between probiotics and host cellular responses, the development of more therapeutics from naturally derived or modified probiotics may be part of our future.

Keywords

cell adhesion; inflammation; intestinal epithelial cell; Lactobacillus rhamnosus GG; probiotics

A surprising characteristic of human beings is the fact that only 10% of cells in the human body are truly human, with all other cells coming from microbiota. The gastrointestinal tract, where the majority of microbiota reside, makes a great contribution to health by establishing the symbiotic relationship between microbiota and the host. The microbe–host interaction is important for regulating host metabolism and biosynthetic pathways, promoting homeostasis, and eliminating toxic substances. Interruption of this relationship has been associated with several pathologic conditions, including inflammatory bowel...
disease, obesity, and late-onset autism. Therefore, manipulation of intestinal bacteria is a potential alternative therapy for disease prevention and treatment.

To explore the broad application of probiotics for health and diseases, well-designed and mechanistically based clinical trials are needed. Likewise, understanding the mechanisms of probiotic action is necessary to better design these clinical trials. Currently, distinct cellular and molecular mechanisms underlying the beneficial effects of probiotics have been revealed by both clinical and basic research, which include blocking pathogenic bacterial effects by production of antibacterial substances and competitive inhibition of pathogen and toxin adherence to the intestinal epithelium, restoring and maintaining the intestinal microbiota, upregulating immune function to improve the ability to fight infections or inhibit tumor formation and downregulating immune responses to inhibit the onset of allergy or intestinal inflammation, and modulating intestinal epithelial homeostasis, such as restitution of damaged epithelial barrier, production of antibacterial substances and cell-protective proteins, and blockade of cytokine-induced intestinal epithelial cell apoptosis (Figure 1). A recent advance in probiotic studies was the discovery of probiotic-derived proteins that interact with host cells. In addition to direct contact with host cells, probiotics generate various products to regulate host cellular responses. The significance of this finding is that applying probiotic-derived products could solve some of the problems associated with the use of live probiotics. First, determining the bioavailability of bacteria in the gastrointestinal tract has been a challenge to understanding efficacy. In addition, the use of live probiotic bacteria raises concerns about biosafety, with several cases of bacterium-associated infections while using probiotic therapy in very young and immunocompromised patients and increased risk of mortality in patients with severe acute pancreatitis. Thus, developing probiotic-derived products as innovative therapeutic reagents has a high promise for clinical application. This review focuses on significant research findings regarding currently identified Lactobacillus rhamnosus GG (LGG)-derived products and their mechanisms of action (see Figure 1). The majority of studies discussed in this review were published after June 2009.

LGG Genes and Gene Products

LGG is a naturally occurring gram-positive bacterium originally isolated from the healthy human intestine. To unravel the biologic function of LGG, genomic analysis has accelerated research toward the molecular basis for probiotic health-promoting activities. A genomic study found the LGG-specific islands that include genes coding for bacteriophage components, sugar metabolism and transport, and exopolysaccharide biosynthesis. One island found in LGG contains genes for three secreted LPXTG-like pilins (spaCBA) and a pilin-dedicated sortase. Functional analysis of SpaC showed that it is on the surface of LGG and is essential for the interaction between LGG and the mucus and maintaining LGG’s ability to persist in the human intestinal tract. Genome-scale analysis of LGG also identified a gene cluster that encodes the enzymes and regulatory and transporter proteins for the different steps in the biosynthesis of long, galactoserich extracellular polysaccharides. Mutation of the welE gene, which encodes the glycosyltransferase, led to deprivation of extracellular polysaccharide molecules to cause less shielding of adhesins and
increased adherence and biofilm formation capacity of LGG. The results from these genomic studies provide great promise for the discovery of novel probiotic effector molecules.

To link the genome and the transcriptome to potential biologic functions, a comprehensive proteomic study was performed using the gel liquid chromatography-mass spectrometry/mass spectrometry method to analyze LGG and Lc705 proteins. More than 1,600 high-confidence proteins were found, covering nearly 60% of the predicted proteomes in LGG and Lc705, including a high number of lipoproteins, integral membrane proteins, peptidoglycan-associated proteins, and proteins predicted to be released into the extracellular environment. This study also revealed the expression of more than 90 proteins in LGG and 150 proteins in Lc705 that lack evolutionary counterparts in the other strain, which were involved in biofilm formation, phage-related functions, reshaping the bacterial cell wall, and immunomodulation.

Another two proteome analyses using a two-dimensional difference gel electrophoresis approach demonstrated the effects of factors in the environment on the proteome responses of LGG. The analysis showed that the growth of LGG in industrial-type whey-based medium increased the relative abundance of proteins involved in purine biosynthesis, galactose metabolism, and fatty acid biosynthesis. However, the growth of LGG in laboratory medium resulted in an increase in the amount of proteins involved in translation and the general stress response, as well as pyrimidine and exopolysaccharide biosynthesis. Moreover, several enzymes of the proteolytic system of LGG were demonstrated as LGG culture medium-dependent in production. Thus, the pattern of protein production is greatly affected by growth conditions. Further studies regarding pH-dependent protein production demonstrated that the acidic condition upregulates all proteins predicted as surface antigen and adenosine triphosphate (ATP) synthase genes. However, nucleotide biosynthesis and protein synthesis were decreased under this condition. In addition, phosphorylation of glycolytic enzymes, which mediates central cellular pathways in LGG, was found to be enhanced by the acidic condition. Thus, this evidence demonstrates the fundamental effects of culture conditions on the characteristics of LGG’s biologic activity.

Cytoprotective Effects of LGG-Derived Products on Intestinal Epithelial Cells

The intestinal epithelium is critical for maintaining normal intestinal function through formation of a regulated physiologic barrier against pathogenic microbes and detrimental substances in the intestinal lumen and actively regulating immune function and maintaining intestinal homeostasis. Disruption of the integrity of this monolayer occurs in several diseases, such as inflammatory bowel disease and some bacterial and virus infections. One of the first targets of probiotic action is the intestinal epithelial cell, and substantial evidence indicates that probiotic bacteria, including LGG, stimulate intestinal epithelial cellular responses, including restitution of damaged epithelial barrier, production of antibacterial substances and cell-protective proteins, and blockade of cytokine-induced intestinal epithelial cell apoptosis.
A significant discovery in the characterization of LGG-derived products was the cloning and purification of p40 and p75, two LGG-derived soluble proteins from LGG culture broth. Both of these proteins were reported to prevent cytokine-induced intestinal epithelial damage and apoptosis\textsuperscript{16,17} and to reduce hydrogen peroxide disruption of epithelial barrier function.\textsuperscript{18} p40 exerts more potent effects than p75. The molecules exhibit differing biologic and signaling potency, such that p40 can stimulate two- to threefold greater Akt activation and inhibition of apoptosis than p75, even at fivefold lower molar concentration than p75. In addition, p40 may be a major protein responsible for the cellular effects regulated by probiotic soluble proteins. \textit{Lactobacillus casei}–conditioned medium contains the same amount of p40 but a very low amount of p75 compared to LGG-conditioned medium. However, \textit{L. casei}–conditioned medium exerts the same effects of Akt activation and inhibition of apoptosis as LGG-conditioned medium.\textsuperscript{16}

Recent studies revealed mechanisms of p40 regulation of cellular responses in intestinal epithelial cells and a protective and therapeutic role in dextran sulfate sodium (DSS)-induced intestinal epithelial injury and acute colitis and oxazolone-induced Th1 cytokine–driven chronic colitis in mice. p40 has been shown to activate epidermal growth factor (EGF) receptor and its downstream target, Akt, leading to amelioration of cytokine-induced apoptosis and disruption of the epithelial barrier in intestinal epithelial cells.\textsuperscript{19} Src and metalloprotease may serve as upstream signals to release ligands as the mechanism for p40 transactivation of EGF receptor in intestinal epithelial cells (unpublished data, 2012). Furthermore, specific delivery of p40 to the colon using special hydrogel beads to protect p40 from degradation prevented and treated colon epithelial cell injury and inflammation in these colitis models in an EGF receptor–dependent manner (Figure 2).\textsuperscript{19}

Given that p40 and p75 represent promising LGG-derived proteins for potential clinical application, several groups have further characterized the structure and function(s) of p40 and p75 in LGG and the LGG-related strain \textit{L. casei}. Both p40 and p75 in \textit{L. casei} BL23 were found at the bacterial cell surface and secreted in the culture medium. Recombinant p40 and p75 proteins bound to mucin, collagen, and intestinal epithelial cells and were able to hydrolyze the muropeptides from \textit{L. casei} cell walls.\textsuperscript{20} Further characterization of p40 and p75 confirmed that both have \( \omega \)-glutamyl-L-lysyl endopeptidase activity.\textsuperscript{21,22} More studies showed that p75 is O-glycosylated with ConA-reactive sugars at the two serine residues in LGG. Glycosylation of p75 plays a role in stability and protection against proteases but does not mediate peptidoglycan hydrolase activity or activation of signaling, such as Akt activation in epithelial cells.\textsuperscript{23} Functional analysis of the p40 and p75 proteins from \textit{L. casei} BL23 and LGG suggested that p75 is involved in bacterial separation. But no bacterial phenotype was found by inactivation of p40.\textsuperscript{20–22}

In another example of LGG-derived products demonstrating cytoprotective roles in intestinal epithelial cells, LGG-conditioned medium prevented radiation-induced small intestinal epithelial injury. Gavage of LGG or LGG-conditioned medium exerted a preventive role in improving crypt survival and reducing epithelial apoptosis at the crypt base in the small intestine of mice following radiation treatment. These effects were dependent on Toll-like receptor (TLR)2 and cyclooxygenase (COX)-2 signaling, leading to
repositioning of constitutive COX-2-expressing mesenchymal stem cells to the crypt base.\textsuperscript{24} Although no specific factor in LGG-conditioned medium was identified in this study, these results further support the possibility of using LGG-derived products as potential radioprotective agents.

The intestinal epithelium is constantly exposed to high levels of genetic material such as bacterial deoxyribonucleic acid (DNA). Previous studies have reported the immunoregulatory effects of DNA from the probiotic VSL\#3 mixture on humans and mice.\textsuperscript{25} LGG DNA has also been shown to enhance expression of TLR9, which was associated with attenuation of tumor necrosis factor (TNF)-induced nuclear factor \(\kappa\)B (NF-\(\kappa\)B) activation and interleukin-8 expression in intestinal epithelial cell lines, HT-29 and T84 cells, through downregulation of I\(\kappa\)B\(\alpha\) degradation and p38 phosphorylation. LGG DNA also showed an inhibitory effect on TNF-induced transepithelial resistance reduction.\textsuperscript{26}

Two previously published articles also indicated that products in LGG culture media promoted cytoprotective protein production by intestinal epithelial cells\textsuperscript{27} and inhibited lipopolysaccharide-induced TNF production in macrophages.\textsuperscript{28} Taken together, these findings reveal a previously unrecognized role for probiotic-derived products in regulating intestinal homeostasis and provide strong evidence to support the role of soluble products produced by probiotics in preventing and/or treating intestinal inflammatory diseases.

**LGG-Derived Proteins for Adhesion to Intestinal Mucosa**

One of the LGG characteristics responsible for its health-benefiting properties is the prolonged residence in the gastrointestinal tract, which is likely dependent on adherence to the intestinal mucosa. However, the molecular mechanism of LGG adhesion to the intestinal mucosa was not understood until recent findings from the genomic analysis of LGG showing the spaCBA gene cluster, the pilus-associated SpaC pilin, and another putative pilus cluster, spaFED, in LGG genome sequence.\textsuperscript{8} Pili are heteromeric and proteinaceous surface appendages that are present in numerous gram-positive bacteria. These proteins mediate adherence between pathogenic and nonpathogenic bacteria and their host cell targets. Pili produced by LGG have been recently characterized by several studies. LGG-produced SpaCBA pili were shown as heterotrimeric protrusions, with SpaA subunit as the shaft-forming major pilin, SpaB pilins at pilus bases, and SpaC adhesin present along the whole pilus length. SpaB mediates termination of pilus assembly, and SpaC plays a role not only in long distance and intimate contacting with host tissue but also in providing mucusbinding strength.\textsuperscript{29} Functional analysis of LGG-produced pili suggested that the SpaCBA pilus plays a key role in efficient adherence to an intestinal epithelial cell line, Caco-2 cells, biofilm formation, and inhibiting lipoteichoic acid–induced interleukin-8 production in Caco-2 cells.\textsuperscript{30} In addition to pili, another LGG surface protein involved in mucosal adhesion has been reported. A recombinant protein was generated based on the LGG gene that exhibits homology with a known mucus-binding domain. This protein is distributed throughout the cell surface of LGG and participates in the adhesive interaction between LGG and mucus. Thus, it may serve as an active mucus-specific surface adhesin involved in pilus-mediated mucosal adhesion.\textsuperscript{31}
Production of Antibacterial Substances by LGG and Other *Lactobacillus* Strains

The production of antibacterial substances is a well-known mechanism underlying the effect of probiotics on bacterial infection. Bacteriocin is such a substance produced by probiotics. Bacteriocins are a heterogeneous family of small, heat-stable peptides with potent antimicrobial activity through interfering with cell wall structure and biosynthesis, forming pores in the target bacterial membrane, and permeablizing membranes. Gram-positive bacterium-produced bacteriocins have a relatively narrow spectrum of activity and are mostly toxic to other gram-positive bacteria, such as Lactococcus, Streptococcus, Staphylococcus, Listeria, and *Mycobacterium*. An example of this case is the finding that *Lactobacillus salivarius* UCC118 produced a bacteriocin in vivo that was required for *L. salivarius* UCC118 protection of mice against an invasive foodborne pathogen, *Listeria monocytogenes*, infection. Recent studies further characterized the production of bacteriocin by seven *L. salivarius* isolates of human and porcine intestinal origin. The genome-wide comparison of these strains showed a highly conserved megaplasmid-borne gene cluster involved in the regulation and secretion of bacteriocins in these strains. Although there is no direct evidence to show the production of bacteriocins by LGG, other antibacterial products secreted by LGG have been reported. Seven peptides were isolated from LGG-conditioned media, which showed anti-gram-negative and anti-gram-positive bactericidal activity. These small peptides showed various degrees of antibacterial activity, with NPSRQERR showing the most potent antibacterial effect. In addition to the antibacterial effects, probiotics showed inhibitory effects on pathogen-derived toxins. Exopolysaccharides produced by LGG, as well as the bifidobacterial strains *animalis* subsp. *lactis* A1, IPLA-R1, and *longum* NB667, blocked the cytotoxic effect of *Bacillus cereus* extracellular factors on colonocytes, such as Caco-2 cells, and the hemolytic activity of streptolysin-O on rabbit erythrocytes. Another preliminary experiment suggested that LGG played an antimicrobial role against *Salmonella* 1344 in vitro, which was mediated by the production of lactic acid and the secretion of non-lactic acid molecules. Regulation of lactic acid production by *Lactobacillus* has also been studied. Two proteins, ldhD1 and ldhD2, that might be responsible for D-lactic acid formation were screened from the genome database of LGG, and the coding genes of these two proteins were cloned in *L. rhamnosus* CASL for generating the recombinant proteins. Both of these two recombinant proteins showed the ability for D-lactic acid formation. Thus, further studies are needed to elucidate the requirement of these two proteins for lactic acid formation by LGG and their effects on the antibacterial response.

LGG for Disease Prevention and Treatment

LGG has been widely used in the production of yogurt as a nutritional supplement. In the field of probiotic research, LGG is one of the best-studied probiotics in clinical trials. It has been previously reported that LGG exerts effects on treating and/or preventing several
disorders, including ulcerative colitis, diarrhea, and atopic dermatitis. Recent studies revealed LGG’s beneficial effects on the prevention and treatment of more diseases.

A meta-analysis of three randomized, controlled trials showed that the administration of LGG to children for the duration of hospital stay was associated with significantly lower rates of health care–associated diarrhea and symptomatic Rotavirus gastroenteritis compared to placebo. Another randomized, single-blind, placebo-controlled study showed that LGG supplementation temporarily eliminated the vancomycin-resistant enterococci carrier state and increased gastrointestinal counts of Lactobacillus spp. in children. LGG treatment has also been shown to reduce abdominal pain in children with functional gastrointestinal disorders, such as irritable bowel syndrome.

The interest in the potential application of probiotics for obesity through regulation of the intestinal microbiome is under investigation. Clinical studies regarding the perinatal nutritional environment on the health of the mother and child showed that LGG and B. lactis Bb12 intervention for nonobese pregnant women reduced the frequency of gestational diabetes mellitus and larger birth size. Furthermore, this clinical effect was suggested to be mediated through improving glucose tolerance and inducing quantitative insulin sensitivity in pregnant women, which suggests that a probiotic approach is worthy of further consideration for the prevention of childhood obesity.

The antitumor effect of LGG is also a promising finding from studies using several animal models. Administering live or lyophilized LGG significantly increased tumor regression in a murine orthotopic model of bladder cancer compared to bacillus Calmette-Guérin immunotherapy. LGG therapy increased lymphotactin, reduced E-selectin and pro–matrix metalloproteinase (MMP)-9, and downregulated vascular endothelial growth factor (VEGF)-D to levels comparable to those of healthy bladders in tumor-bearing mice, which indicates the potential inhibitory effects of LGG on metastasis-related and tissue-remodeling enzymes. LGG also recruited large numbers of neutrophils and macrophages to the tumor site, which indicates the immunoregulatory effects of LGG in tumorigenesis. Further studies indicated that the mechanism was through neutrophil-stimulated dendritic cell maturation, resulting in increased cytotoxic T lymphocyte activity against cancer cells.

Another example is from studies to investigate aflatoxin-B1 (AFB1)-induced liver carcinogenesis in rats. The LGG and L. casei strain Shirota fermented milk alone or in combination with chlorophyllin, an antioxidant agent, showed a significant hepatoprotective effect by lowering the levels of thiobarbituric acid–reactive substance, a marker of lipid peroxidation, and enhancing the activities of antioxidant enzymes such as glutathione peroxidase, superoxide dismutase, catalase, and glutathione-S-transferase, indicating a potent protective effect against AFB1-induced hepatic damage by these two probiotics.

These new findings toward understanding of LGG’s clinical efficacy urge more research investigating LGG’s mechanisms of action. Studies to further investigate the mechanisms of LGG stimulation of ERK activity via interaction with formyl peptide receptors (FPRs) in intestinal epithelial cells indicate that LGG activates reactive oxygen species (ROS) signaling in an FPR-dependent manner and define a mechanism by which cellular ROS influences the ERK pathway through a redox-sensitive regulatory circuit.
cytokine production by probiotic bacteria, including LGG, has been widely known. A recent report revealed new evidence that LGG and *Streptococcus thermophilus* induce expression of suppressor of the cytokine signaling 3 gene, which mediates the expression of proinflammatory cytokine, directly and indirectly through interleukin-10 in human primary macrophages.48

Thus, the results from both clinical and basic studies widen the possible applications of LGG for disease prevention and treatment. They also support the idea of exploring products derived from LGG as targets for clinical application of LGG-derived products.

**Future Studies**

Promising health benefits and the efficacy of probiotic-derived products for preventing and treating a variety of diseases have attracted increasing attention to inclusion of these agents as functional foods. However, compared to genomic analysis–predicted LGG functional proteins, the number of identified LGG-derived products is limited. It also should be noted that more mechanistic evidence that supports the effects of these products on cellular responses, leading to determining the end points of diseases, is needed. Given that the correct combination and concentration of probiotic-derived products may improve the efficacy of this approach for the prevention and treatment of human diseases in the future, there are several important details of clinical application of these products that need to be addressed.

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**References**


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Figure 1.
Regulation of host homeostasis by probiotics and probiotic-derived products. Probiotics and/or probiotic-derived products exert several beneficial effects on host responses. These include promoting intestinal epithelial homeostasis by increasing survival, barrier function, and cytoprotective responses (1), defining the balance between necessary and excessive defense immunity by increasing innate immunity (2), upregulating antiinflammatory cytokine production (3) and inhibiting proinflammatory cytokine production (4), blocking pathogenic bacterial effects by producing antibacterial substances (5), and competing with pathogens for binding to epithelial cells (6). Probiotic-derived products also enhance adherence of probiotics to the epithelial cells (7). DC = dendritic cell; Hsp = heat shock protein; IFN = interferon; IL = interleukin; M = M cell; TH = T helper; TNF = tumor necrosis factor.
Figure 2.
Regulation of colitis by p40, a *Lactobacillus rhamnosus* GG (LGG)-derived protein. p40 stimulates Src activity, which leads to activation of matrix metalloproteinases (MMPs). MMPs are proteolytic enzymes that induce the release of epidermal growth factor receptor (EGFR) ligands for transactivation of EGFR and its downstream target, Akt, in the colon epithelial cells. Increased apoptosis and disruption of barrier function in the intestinal epithelial cells are two pathologic factors involved in colitis induced by dextran sulfate sodium (DSS), oxazolone, and 2,4,6-trinitrobenzene sulfonic acid (TNBS). Activation of EGFR and Akt by p40 to prevent apoptosis and maintain intestinal integrity serves as a mechanism for p40’s preventive and treatment effects on intestinal inflammation in these mouse models of colitis.